Quantitative evaluation of CART-containing cells in urinary bladder of rats with renovascular hypertension

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Abstract

Recent biological advances make it possible to discover new peptides associated with hypertension. The cocaine- and amphetamine-regulated transcript (CART) is a known factor in appetite and feeding behaviour. Various lines of evidence suggest that this peptide participates not only in control of feeding behaviour but also in the regulation of the cardiovascular and sympathetic systems and blood pressure. The role of CART in blood pressure regulation led us to undertake a study aimed at analysing quantitative changes in CART-containing cells in urinary bladders (UB) of rats with renovascular hypertension. We used the Goldblatt model of arterial hypertension (two-kidney, one clip) to evaluate quantitative changes. This model provides researchers with a commonly used tool to analyse the renin-angiotensin system of blood pressure control and, eventually, to develop drugs for the treatment of chronic hypertension. The study was performed on sections of urinary bladders of rats after 3, 14, 28, 42 and 91 days from hypertension induction. Immunohistochemical identification of CART cells was performed on paraffin for the UBs of all the study animals. CART was detected in the endocrine system, including various types of endocrine system (DNES). These cells produce active substances that affect the coordination of clotting factors. There are genetic predispositions and disorders of complex mechanisms regulating blood pressure underlining both primary and secondary hypertension. It is postulated that the nervous and endocrine systems, kidneys and local factors regulating the tension of muscles in blood vessels are responsible for regulating the pressure.1 The results of research in recent years also suggest a considerable role of the endocrine system, including various types of highly specialized, diffusely located receptor-effector cells included in the diffuse neuroendocrine system (DNES). These cells produce active substances that affect the coordination of a wide range of functions of organs and are direct regulators of homeostasis in the body. However, they can simultaneously play roles as mediators in several circulatory disorders, including renovascular hypertension.2

Introduction

Renovascular hypertension is a common disorder and an illness with an added social dimension.1 It sporadically occurs as an isolated condition, however more often it occurs in combination with other illnesses, especially metabolic disorders, including disorders of lipid and lipoprotein management, diabetes, obesity, hyperuricemia and changes in the quantitative composition of cloting factors. There are genetic predispositions and disorders of complex mechanisms regulating blood pressure underlining both primary and secondary hypertension. It is postulated that the nervous and endocrine systems, kidneys and local factors regulating the tension of muscles in blood vessels are responsible for regulating the pressure.2 The results of research in recent years also suggest a considerable role of the endocrine system, including various types of highly specialized, diffusely located receptor-effector cells included in the diffuse neuroendocrine system (DNES). These cells produce active substances that affect the coordination of a wide range of functions of organs and are direct regulators of homeostasis in the body. However, they can simultaneously play roles as mediators in several circulatory disorders, including renovascular hypertension.2 Among the active substances secreted by DNES cells, there is the cocaine and amphetamine regulated transcript (CART), produced and secreted in the hypothalamus and in other tissues in many vertebrates.3,4,11 Despite considerable progress made in the area of structure and physiological description of this factor, its role in the pathogenesis of human hypertension is controversial. The development of techniques in molecular biology and histochemistry has enabled research on the distribution and precise tissue localization of the CART peptide in many organs. The principal site of CART synthesis is in areas of the brain, involved in the control of blood pressure.5,11 CART is mainly known for its role in feeding anomalies, which result in hypophagia. This concerns a wide range of biological activities. Among others, CART has neurodegenerative properties after a stroke and is related to blood circulation and renovascular hypertension.10,12-14 Central administration of CART peptide increases systemic blood pressure15 and blocks phenylephrine-induced bradycardia.16 There is only limited information concerning the influence of CART on heart rate, blood pressure, stress and secretion of adrenal hormones. There is both anatomical and physiological evidence available that CART is engaged in hypothalamus - pituitary gland - adrenal gland (HPA) regulation. The HPA axis represents one of the main limbs of an adaptive system, which maintains the basal and stress-related homeostasis in vertebrates.17 It is the main component of the neuroendocrine system responsible for the coordination of many systems, including the cardiovascular system.17,18 The fact that CART is found at the level of, HPA as well as coexisting with peptides with pressor properties such as leptin, neuropeptide Y (NPY) or calcitonin gene related peptide (CGRP),19 can indicate a potential role of CART in physiological regulation of blood pressure. The presence of CART in the HPA axis is stric-
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Measuring by the tail-cuff method and the arte-
men procedures, and the third group (n=40) included rats
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days following the clamping of the left renal
sence of the duration of hypertension on the
mice, which activated the sympathetic nervous sys-
denzyme activity of CART was verified by
presented free of the renal vein. The renal artery was
ly related to activation of the sympathetic nerv-
and noradrenaline, which further

ty protocols

Materials and Methods

Animal protocols

The study included 6-week-old young male
Wistar rats (*Rattus norvegicus*). Their body
weight at the beginning of the experiment was
160-180 g (mean body weight: 170±10 g). The animals
were maintained at 22±1°C with an
alternating 12:12-hour light-darkness cycle.
Food and water were available *ad libitum*.
Animal protocols were reviewed and approved by
the Senate Committee for Oversight of
Experiments on Humans and Animals, Medical
University in Bialystok, Poland (Resolution no.
49/2009 on 30.09.2009, concerning application
no. 2009/45).

The animals were divided into 3 groups; the
first group (n=15) included sham-operated
(SO) rats, the second group (n=15) included
control rats not undergoing any surgical proce-
dures, and the third group (n=40) included rats
with experimentally induced renovascular hypertension (2K1C). All procedures and meas-
urements were performed at the same time of
the day. After a 7-day acclimatization period,
each rat had its systolic blood pressure (SBP)
measured by the tail-cuff method and the arte-
rial pressure was evaluated by using a Student
Oscillograph Rat Tail Blood Pressure Monitor
Kit (Harvard Apparatus, Holliston, MA, USA).

### Description of the experiment

Induction of experimental hypertension was
performed according to procedure developed
by Goldblatt et al. After the rats were anaesthet-
ised by exposure to pentobarbital (40
mg/kg, i.p.), a 3-cm retroperitoneal flank inci-
section was performed in sterile conditions. The
left kidney was exposed and the renal artery was
carefully dissected free of the renal vein. The
renal artery was then partially occluded by
placing a standardized silver clamp with an
internal diameter of 0.22 mm on the vessel. The
wound was closed with a running 3-0 silk
suture. The sham-operated rats (n=15) under-
going surgical procedures, except that
a clip was not applied to the renal artery. After
the surgery, the rats were kept in separate
cages. After 3, 14, 28, 42 and 91 days from the
renal artery clamping procedure, all rats were
weighed and the systolic arterial pressure was
measured by the tail-cuff method. After this
time, all the 2K1C rats (n=40) developed stable
hypertension (mean blood pressure values

### Histology procedure

After 3, 14, 28, 42 and 91 days following the
clamping procedure and induction of renova-
cular hypertension, all the animals were put
into a state of deep anesthesia with pentobarbital (50
mg/kg body weight). After cardiac arrest, fragments of the bladder wall were col-
lected from the animals, always from the same
place (body of urinary bladder). Sections of the
wall, sampled in the same way as from the
experimental group, were collected from rats
not undergoing any procedures to provide com-
parative material.

Tissues were fixed (72 h at room tempera-
ture) by immersion in 4% buffered formalde-
hyde and, following dehydration, were embed-
ded in paraffin wax. The sections were cut 4
µm thick and attached to FLEX IHC microscope
slides (K8020, Dako, Glostrup, Denmark).

### Immunohistochemical protocol

The identification and visualization of CART
in neuroendocrine cells (NE) was performed
based on the EnVision method according to
Herman and Elfont using a commercial polyclonal antibody against CART. For antigen
retrieval, immunostaining was performed
using the below protocol. The sections were
deparaffinized in xylene and hydrated in a
series of alcohols with decreasing concentra-
tion. The sections were subjected to pre-treat-
ment in a pressure chamber heated for 1 min
at 21 psi at 125°C, using Target Retrieval
Solution, pH of 9.0 (S 2367, Dako). After being
cooled to room temperature, these sections
were incubated with Peroxidase Blocking
Reagent (S 2001, Dako) for 10 min to block
endogenous activity. The sections with the pri-
mary antibody for the CART peptide (rabbit
clonal CART antiserum, No H-003-61, pur-
chased at the Phoenix Pharmaceuticals, Inc.
Mountain View, CA, USA) were diluted
(1:10,000) in antibody diluents (S 8899, Dako).
The slides were incubated overnight at 4°C in
a humidified chamber with the diluted anti-
body, followed by incubation with a secondary
rabbit antibody (conjugated to horseradish
peroxidase-labeled polymer) (EnVision+ Kit
HRP Rabbit K4011, Dako) for 1 h. The bound
antibodies were visualized by 1-min incuba-
tion using the 3,3’-diaminobenzidine (DAB)
substrate chromogen. All of the sections were
finally stained with Vector QS hematoxylin
(H-3404; Vector Laboratories, Inc., Burlingame,
CA, USA), mounted and evaluated under a
light microscope. Appropriate washing with
Wash Buffer (S 3006, Dako) was performed
between each step.

The specificity test performed for the CART
antibody included: negative control, where
the antibodies were replaced by normal rabbit
serum (Vector Laboratories; Burlingame, CA,
USA) at the respective dilution. Additionally,
positive control was carried out for the specific
tissue, as recommended by the producer
(for our research we used rat hypothalamus). The
stained preparations were analyzed under an
Olympus BX 41 microscope. Two sections of
each fragment of the urinary bladder from
each animal were studied. The cells containing
CART were localized and observed in 5 ran-
domly selected fields of vision (0.785 mm²) at
a 200x zoom (20x objective and 10x eye piece)
of the analyzed urinary bladder section area.

### Statistics

The rats were divided into 2 groups: study
control and each of these 2 into 5 further
groups (1-5) according to the duration of the
experiment. The duration was measured in
days: 1st group, 3 days; 2nd group, 14 days; 3rd
group, 28 days; 4th group, 42 days; 5th group, 91
days. After completion of the experiment, the
number of CART-immunoreactive (CART-IR)
cells in the all layers of the urinary bladder wall
was determined for each rat in the particular
groups. The parameters were determined at
two decimal places accuracy. The determined
numbers of endocrine cells (in the individual
layers of wall urinary bladder), as well as the
duration of illness, were subjected to statistical
analysis.

### Statistical analysis

All the collected data were analyzed statisti-
cally using STATISTICA Version 10.0 (StatSoft
Polksa Inc., Kraszewskiego 36, 30-110 Kraków,
Poland). The numbers of NE cells were ana-
alyzed using the Mann-Whitney test for inde-
pendent variables. When testing statistical
hypotheses, P<0.05 was assumed as the level of significance.

Results

No significant differences between the two control groups of rats were observed in the obtained results of the study. Only the results concerning sham-operated animals were taken into account. Blood pressure monitoring showed that, at week 6 of the experiment the blood pressure in the renovascular hypertensive rats stabilized at 162.6±2.19 mmHg, while in control groups remained at 120.2±5.89 mmHg. Hypertensive rats were gaining weight at a similar rate as normotensive animals. Immunohistochemical (IHC) staining enabled the identification of CART-IR cells in the entire urinary bladder wall, both in the normotensive and hypertensive group during all the weeks of induced hypertension. Microscopic observations allowed the location of CART-positive cells, as well as their numbers in relation to the duration of the disorder. Analyzing the location of endocrine cells, in all experimental groups, the most numerous CART-IR cells were found in the urinary bladder muscularis layer and the submucosa (Figure 1A), whereas they were only a few observed in the transitional epithelium and occasionally under urothelium (Figure 1B).

The observed cells were predominantly individually scattered or formed small groups of 2-3 (Figure 1C), sporadically 5-6 cells (Figure 1D).

Quantitative evaluation of CART-IR cells

Statistical analysis confirmed the intuition-al view that, in the first stage of the hypertension, the greatest changes included increased activity of NE cells with a significant increase in their mean number in the experimental group (Table 1). An increase of nearly 50% was observed three days after ligation of the left renal artery in the urinary bladder walls of the rats from the experimental group (Figure 2A), in comparison with their counterparts in the control group (Figure 2B).

In the next experimental group, i.e. the two-week one, two weeks after the ligation procedure, a rise in the numbers of NE cells secreting CART was also observed in the control group, though lower when compared with the 1st study group. In the 3rd study group (28 days), endocrine cell density (23.88±6.06) in the animals with renovascular hypertension only slightly differed from the 1st study group. In the analysis, the greatest population of CART-IR cells (in normotensive and hypertensive rats) in the experimental model of unilateral renal artery stenosis was observed after 42 days in the experimental group (Table 1).

Table 1. Number of CART-immunoreactive cells in the urinary bladder of control and the 2K1C rats at different times of renovascular hypertension (mean ± SD).

<table>
<thead>
<tr>
<th>Time after artery clipping procedure</th>
<th>Group of rats</th>
<th>Number of CART-IR cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>Control</td>
<td>13.67±2.12</td>
</tr>
<tr>
<td></td>
<td>2K1C</td>
<td>23.63±4.38*</td>
</tr>
<tr>
<td>14 days</td>
<td>Control</td>
<td>14.67±4.24</td>
</tr>
<tr>
<td></td>
<td>2K1C</td>
<td>22.38±4.06*</td>
</tr>
<tr>
<td>28 days</td>
<td>Control</td>
<td>15.33±0.71</td>
</tr>
<tr>
<td></td>
<td>2K1C</td>
<td>23.88±6.06*</td>
</tr>
<tr>
<td>42 days</td>
<td>Control</td>
<td>18.00±1.41</td>
</tr>
<tr>
<td></td>
<td>2K1C</td>
<td>25.50±5.68*</td>
</tr>
<tr>
<td>91 days</td>
<td>Control</td>
<td>16.00±2.83</td>
</tr>
<tr>
<td></td>
<td>2K1C</td>
<td>21.13±7.06*</td>
</tr>
</tbody>
</table>

Cart-IR, CART-immunoreactive; 2K1C, Goldblatt’s model of hypertension – two-kidney, one clip model; *P>0.05.
days of illness in comparison with the other groups included in the experiment. In this group, much larger clusters of cells were also noticed in comparison with the other study groups. The clusters consisted of up to 5-6 cells and were mainly located in the muscularis layer (Figure 3A). Numerous cells were also reported in the urothelium only in this study group. In the mucosa innominate active cells were observed in the surface cell layer of transitional epithelium (Figure 3B).

Another substance that has influence on hypertension, and still constitutes a subject of many discussions, is CART. It is principally known for its role in feeding but, as shown in a number of studies, its role is not limited only to hypophagia as it has an impact on many organs and systems.10,12 CART is a peptide involved in a number of physiological processes including modulation of the HPA axis and cardiovascular regulation.13,16-20 Its function as a factor in the development of hypertension, despite numerous studies, remains controversial. Arguments in favour of CART influence on the cardiovascular system are provided in studies of Hwang et al.,21 Matsumura et al.,22 and et al.23 In their experiments, after direct infusion of CART to cerebral ventricles, Matsumura et al.10 noted an increase in mean blood pressure and plasma glucose concentration. On the other hand, lliff et al.24 reported that, in isolated cerebral arterioles, CART peptide (CARTp) acts directly to produce endothelium-dependent constriction via the endothelin signaling pathway.

Visualization of innervation changes, together with the development of nervous structures secreting CART in the urinary bladder of rats, was performed by Zvarova et al.25 However, the results did not concern normotensive animals. The knowledge relating to the identification and distribution of CART in pathological states caused by hypertension has so far been limited only to specific organs of the alimentary tract,1 as well as the bladder and ureters.1

Kasacka and Piotrowska postulated the connection of CART with hypertension and used an IHC method in the gastrointestinal tract (GI tract) of rats in an experimental model of unilateral renal artery stenosis. After 42 days from inducing the condition, the researchers measured the number of NE cells and structures secreting CART in comparison with normotensive animals. The results of the observations were in accordance with the prognosis because the hypertension caused a significant increase in the mean number of CART-IR cells and structures. A close connection between hypertension and the scattered nervous system has also been proved in the region of the lower urinary tract in hypertensive rats. Besides the abovementioned studies, no information has been found on the subject of dynamics of changes in CART-IR cells in the urinary tract of hypertensive animals in relation to the duration of the illness. The connection between the duration of hypertension and the number of DNES cells has been documented only in studies of Kasacka and Arciszewska who analyzed the dynamics of changes in the number of NE cells under the influence of 3-, 14-, 28-, 42-, and 91-day hypertension. The aim of their studies was to examine the distribution, morphology and dynamics of changes of calcitonin gene related peptide-containing cells in the lungs of rats in the two-kidney, one-clip (2K1C) renovascular hypertension model. The authors proved that there is a connection between hypertension, the number of NE cells containing CGRP and the duration of hypertension. Analyzing the results, the greatest intensity of NE cells in the lungs of rats was noticed 14 days after the surgical procedure.3

In this paper, the presented data concern the duration of hypertension and the number of CART secreting cells in the urinary bladder of rats in a renovascular hypertension model. After 3 days, the number of NE cells in the urinary bladder walls of the rats that underwent the surgical procedure of inducing renovascular hypertension significantly increased. This pattern was not described by Kasacka and Arciszewska in rat lungs after 2K1C. Three and 28 days after clamping the left renal artery, the number of cells reached comparable levels in the control and study group. However, it was lower in the study group after 3 days since hypertension had been induced. In contrast, Kasacka and Arciszewska observed the greatest number of CGRP-IR cells 14 days after the procedure in the lungs of hypertensive rats. In our observations, the highest parameters were noted only 6 weeks after hypertension was induced. These and earlier promising data let us assume that the studied peptide may play an active role in the development of renovascular hypertension and related disorders.46

Renovascular hypertension caused by renal artery stenosis in original Goldblatt’s experi-

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**Discussion**

Renovascular hypertension caused by renal artery stenosis may lead to renal hypoperfusion and development of a sequence of changes in hormone and neuropetide secretion, which in consequence leads to hemodynamic disorders. Multiple studies have proved that, in patients, renal artery stenosis in early stages of the illness is an incentive to increasing renin production by the ischemic kidney and, as a result, to increased activity of the HPA axis as well as excessive production of angiotensin II.2,19,22 Among other significant factors in the pathogenesis of hypertension there are active substances correlated with the HPA axis and the sympathetic nervous system. It is assumed that, besides the stimulation of the renal hormonal system, there is an intensified activity of DNES cells, which are diffusely distributed throughout the body and produce pressor substances.23 The group of substances with pressor characteristics, playing a vital role in pathophysiology of hypertension, includes those related to hunger, such as ghrelin, leptin or NPY.4,24

CART-containing cells observed in surface cell layer of the transitional epithelium.

**Figure 3. A) Forty-two-day experimental model of unilateral renal artery stenosis; visible immunohistochemical reaction in numerous CART-containing cells in muscularis layer. B) CART-containing cells observed in surface cell layer of the transitional epithelium. Scale bars: 20 μm.**
der incontinence, frequency and nocturia. Angiotensin II (Ang II), a key element of this system, plays a pivotal role in the pathogenesis of clinical and experimental hypertension, and also modulates fluid balance and the cardiovascular and urinary systems. As a vascular risk factor, hypertension has been reported to play a role in the development of urinary tract symptoms, especially when hypertension increases in CART-IR numbers in the urinary tract, the gastrointestinal tract and the respiratory system. Changes in pituitary adenylate cyclase activating polypeptide expression in urinary bladder pathways after spinal cord injury. Exp Neurol 2005;192:46-59.

In conclusion, CART seems to be a new target of CART-IR numbers in the urinary tract, the gastrointestinal tract and the respiratory system. Changes in pituitary adenylate cyclase activating polypeptide expression in urinary bladder pathways after spinal cord injury. Exp Neurol 2005;192:46-59.

References


